Date: April 2, 1999 Express Mail Label No. EL192626414US

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Attorney's Docket No.: KIR95-01A

Small Molecular Weight TNF Receptor Multimeric Molecule

RELATED APPLICATION(S)

This application is a Continuation of 08/437,533 filed May 9, 1995, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Tumor Necrosis Factor, a pleiotropic cytokine released by activated T cells and macrophages, is expressed as a mature 17 kDa protein that is active as a trimer (Smith, R.A. and Baglioni, C., *J. Biol. Chem., 262*:6951 (1986). Trimeric cytokines such as Tumor Necrosis Factor (TNFα) and the closely related protein lymphotoxin (TNFβ), exert their biological activity by aggregating their cell surface receptors. The TNF trimer binds the receptors on the cell surface causing localized crosslinking of TNF receptors into clusters necessary for signal transduction.

The action of TNFα and TNFβ are mediated through two cell surface receptors, the 55 kDa (p55 TNF-R) and the 75 kDa (p75 TNF-R) receptors. Truncated forms of these receptors, comprising the extracellular domains (ECD) of the receptors, have been detected in the urine and serum as 30 kDa and 40 kDa TNF inhibitory binding proteins (Engelmann, H., *et al., J. Biol. Chem., 265*:1531 (1990)).

TNF is a key mediator in a number of autoimmune and inflammatory diseases such as rheumatoid arthritis, septic shock, cerebral malaria and multiple sclerosis (reviewed in Tracy, K.J. and Cerami, A., *Ann. Rev. Cell. Biol., 9*:317 (1993)). Antagonistic TNF treatment with anti-TNF antibodies and dimeric TNF-receptor-IgG

fusion chimeras have shown promising therapeutic results for a variety of diseases in animal models (Lesslauer, W., *et al.*, *Eur. J. Immunol., 21*:2883 (1991); Kolls, J., *et al.*, *Proc. Natl. Acad. Sci. USA, 91*:215 (1994); Baker, D., *et al.*, *Eur. J. Immunol., 24*:2040 (1994);Williams, R.O., *et al.*, *Proc. Natl. Acad. Sci. USA, 89*:9784 (1993)) and human clinical trials (Elliot, M., *et al.*, *Arthritis and Rheum., 36*:1681 (1993)).

For example, it has been shown that the IgG-Hu p75 TNF-R ECD dimers have a 100-4000 fold higher affinity for TNF over the monomeric counterparts (Lesslauer, W., *et al.*, *Eur. J. Immunol., 21*:2883 (1991); Kolls, J., *et al.*, *Proc. Natl. Acad. Sci. USA, 91*:215 (1994); Butler, D., *et al.*, *Cytokine, 6*:616 (1994)). However, these molecules are large in size, immunogenic and include the Fc portion of the IgG which may interfere with clearance by binding to Fc receptors.

Thus, a need exists for improved TNF inhibitors which are less immunogenic and allow for faster clearance and greater tissue penetration when administered to a host.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that a small molecular weight protein or tumor necrosis factor receptor (TNF-R), built from two or more TNF-R monomers linked via one or more polypeptide bridges or linkers, is active in inhibiting the biological activity of tumor necrosis factor (TNF). In one embodiment the invention relates to a receptor molecule which binds to TNF comprising all or a functional portion of the extracellular domain (ECD) of two TNF-Rs linked via a polypeptide linker. In another embodiment, the invention relates to a receptor molecule which binds to TNF comprising three TNF-Rs linked via two polypeptide linkers. The receptor molecule can include the ECDs of two or more p75 TNF-Rs or the ECDs of two or more p55 TNF-R. The receptor can further comprise a signal peptide of a secreted protein, such as the signal peptide of the extracellular domain of the TNF-R or the signal peptide of a cytokine.

In another embodiment the invention relates to isolated DNA encoding a protein or receptor molecule which binds to TNF, comprising two or more sequences encoding all or a functional portion of the ECD of TNF-Rs linked via one or more sequences encoding a polypeptide linker.

The invention further relates to a method of making a construct which expresses all or a functional portion of the ECD of two or more TNF-Rs linked via one or more polypeptide linkers comprising the steps of: a) obtaining a first vector which expresses all or a functional portion of the ECD of a first TNF-R and a signal peptide of a secreted protein; b) obtaining a second vector which expresses all or a functional portion of an ECD of a second TNF-R; and c) ligating the first vector of (a) with the second vector of (b) via a polypeptide linker. Thus, the first vector of (a) is linked to the second vector of (b) via the polypeptide linker resulting in a construct which expresses all or a functional portion of the ECD of the first TNF-R and all or a portion of the ECD of the second TNF-R linked via a polypeptide linker. The method of making a construct can further comprise one or more vectors which express a second polypeptide linker and all or a functional portion of an ECD of a third TNF-R wherein the ECD of the third TNF-R is linked to the ECD of the second TNF-R via the second polypeptide linker.

The present invention also relates to cells which express a construct which expresses all or a functional portion of the ECD of two or more TNF-Rs linked via one or more polypeptide linkers.

In another embodiment the invention relates to a method of inhibiting the biological activity of TNF in a host comprising administering to the host an effective amount of a receptor molecule which binds to TNF, the receptor comprising all or a functional portion of the ECD of two or more TNF-Rs linked via one or more polypeptide linkers. The invention can further be used in a method of treating a host for a TNF related disease comprising administering an effective amount of the receptor molecule of the present invention to a host.

The present invention also relates to protein or receptor molecules which bind cytokines that bind to receptor molecules comprising more than one subunit (e.g., IL-2 and IL-6 bind to an α or β receptor protein). The ECD of such receptors linked by a polypeptide linker have higher affinity for the cytokine, and, are effective inhibitors of the biological activity of the cytokine. Thus, the receptor comprises all or a functional portion of the ECD of two or more cytokine receptors linked via one or more polypeptide linkers. Furthermore, the receptor is less immunogenic, allows faster clearance and greater tissue penetration in the host upon administration than recombinant immunoglobulin molecules.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a drawing which illustrates the different stages of cloning used to obtain the Hu p75 TNF-R ECD dimer.

Figure 2 is a drawing which illustrates the cloning of the Hu p75 TNF-R ECD dimer into the retroviral vector pBabe Neo used to obtain the plasmid Oscar.

Figure 3 is the expected DNA sequence (SEQ ID NO: 1) and protein sequence (SEQ ID NO: 2) of the Hu p75 TNF-R ECD dimer in which the signal peptide is underlined, the polyglycine linker is boxed, and the putative N-linked glycosylation sites are indicated by a single bar.

Figure 4 is a photograph of a Western blot of the soluble Hu p75 TNF-R ECD dimer.

Figure 5A is a graph of pg/ml TNF versus % cell death illustrating the standard TNF cytotoxic curve from 0.2 pg/ml to 500 pg/ml.

Figure 5B is a graph of dilution versus % protection of the monomeric Hu p75 TNF-R ECD CRIP supernatant (at 3.35 ng/ml) diluted 1:4 to 1:32 incubated with 62.5 pg/ml TNF.

Figure 5C is a graph of dilution versus % protection of the dimeric p75 sf2 protein (at 2.3 ng/ml) diluted 1:4 to 1:128 with 167 pg/ml human TNF.

Figure 5D is a graph of dilution versus % protection of two fold dilutions of concentrated supernatant from Oscar transfected cells (at 0.31 ng/ml) diluted from 1:4 to 1:256 incubated with 62.5 pg/ml TNF (samples were incubated for 1 hour at 37°C and then applied in triplicate to WEHI cells as described by Butler *et al.*, Cytokine, 6:616 (1994).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery of an efficient small molecular weight tumor necrosis factor/lymphotoxin antagonist which is active in inhibiting the biological activity of tumor necrosis factor (TNF). The present invention relates to a receptor molecule which binds to TNF comprising all or a functional portion of the extracellular domain (ECD) of two or more tumor necrosis factor receptors (TNF-Rs) linked via one or more polypeptide linkers. For example, the receptor molecule can comprise the ECDs of two TNF-Rs linked via a polypeptide linker to produce a dimeric TNF-R, as described in Example 1, or the ECDs of three TNF-Rs linked via two polypeptide linkers resulting in a trimeric TNF-R.

The invention also includes isolated DNA encoding a receptor which binds to TNF, comprising two or more sequences encoding all or a functional portion of the ECD of TNF-Rs linked via one or more sequences encoding a polypeptide linker. In a particular embodiment, the isolated DNA of the present invention is the sequence of Figure 3 (SEQ ID No: 1).

As described in Example 1, in the embodiment in which the ECDs of two TNF-Rs are linked via a polypeptide linker, a small molecular weight TNF-R dimer was produced using two TNF-R monomers linked via a 15 amino acid polyglycine-serine bridge and is active in inhibiting the biological activity of TNF. As described in Example 2, this 59 kDa protein has four potential N glycosylation sites, is recognized in

western blots and in the enzyme-linked immunosorbent assay with monoclonal antibodies against the p75 TNF-R.

Although the present invention is exemplified using the ECD from human p75 TNF-R, other ECDs from TNF-Rs can be used, such as the ECD from the p55 TNF-R. Also, functional fragments or portions of the ECD or derivatives thereof (including site mutations such as one or more amino acid deletions, additions and substitutions) are encompassed. The two or more ECDs can also be the same or different. Thus, the receptor molecule of the present invention is capable of binding tumor necrosis factor (TNFα) and lymphotoxin (TNFβ) and the biological activities of TNFα and TNFβ can be inhibited using the receptor molecule of the present invention.

The ECD of the TNF receptors can be derived from a suitable source for use in the present invention. For example, the ECD of the TNF-Rs can be purified from natural sources (e.g., mammalian, more particularly, human), produced by chemical synthesis or produced by recombinant DNA techniques as described in Example 1. In addition, the present invention includes nucleic acid sequences which encode the ECD of a TNF-R, as well as RNAs encoded by such nucleic acid sequences. As used herein, the ECD of the TNF-R refers to fragments and functional equivalents of the ECD of the TNF-R.

The terms "functional portion, fragment or derivative" refer to the portion of the ECD of the TNF-R protein, or the portion of the TNF-R sequence which encodes the ECD of TNF-R protein, that is of sufficient size and sequences to have the desired function (i.e., the ability to bind TNF) (PCT/GB91/01826; WO 9207076). Functional equivalents or derivatives of the ECD of TNF-R include a modified ECD of the TNF-R protein such that the resulting ECD of the TNF-R has the same or similar binding activity for TNF as the natural or endogenous TNF-R ECD, and/or nucleic acid sequences which, for example, through the degeneracy of the genetic code encode the same peptide gene product as the ECD of TNF-R and/or have the same TNF binding activity as described herein. For example, a functional equivalent of the ECD of the

TNF-R can contain a "SILENT" codon or one or more amino acid substitutions, deletions or additions (e.g., substitution of one acidic amino acid for another acidic amino acid; or substitution of one codon encoding the same or different hydrophobic amino acid for another codon encoding a hydrophobic amino acid). See Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience 1989.

The polypeptide linker preferably includes suitable polypeptide linkers which link or ligate the TNF-Rs of the present invention so as to facilitate the highest binding affinity of the TNF trimer to the ECDs of the receptor molecule described herein. That is, the polypeptide linker of the present invention is of a length and composition which allows binding of the TNF trimer to the receptor of the present invention to occur to its greatest extent. Thus, preferred polypeptide linkers provide minimal steric hindrance to binding of TNF to the receptor molecule (e.g., glycine preferred), minimal immunological reaction and maximal solubility of the receptor molecule. The polypeptide linker can be from about 10 to about 30 amino acids in length, preferably between about 10 to about 20 amino acids. In one embodiment, the polypeptide linker is about 15 amino acids in length, as described in Example 1. In addition, the composition of the polypeptide linker can be for example, a polyglycine-serine linker, a polyglycine-leucine linker, polyglycine-alanine linker and a polyglycine-threonine linker.

The receptor molecule of the present invention can further comprise a signal peptide of a secreted protein to direct expression of the receptor of the present invention. A suitable signal peptide of the present invention includes the signal peptide of the ECD of the TNF-R or the signal peptide of a cytokine. Functional equivalents of the signal peptides of the present invention are also encompassed by the present invention. Functional equivalents of the signal peptide include a modified signal peptide of a secreted protein such that the resulting signal peptide has the same secretion activity as the non-modified signal peptide. Functional equivalents also include nucleic acid

sequences which through the degeneracy of the genetic code encode the same signal peptide as known signal peptides of secreted proteins and have a similar secretion activity.

Thus, the order of the components of the receptor described herein can be: all or a functional portion of a first ECD of a TNF-R, a first polypeptide linker, and all or a functional portion of a second ECD of a TNF-R in one embodiment. In another embodiment the order of components can be: all or a functional portion of a first ECD of a TNF-R, a first polypeptide linker, all or a functional portion of a second ECD of a TNF-R, a second polypeptide linker, and all or a portion of a third ECD of a TNF-R. In addition, in either embodiment, the order of components can begin with a signal peptide. The receptor molecule links the components through peptide bonds and is preferably the result of a single recombinant expression unit.

The invention further relates to a method of making a construct which expresses all or a function portion of the extracellular domain of two or more TNF-Rs linked via one or more polypeptide linkers comprising the steps of: a) obtaining a first vector which expresses all or a functional portion of an ECD of a first TNF-R and a signal peptide of a secreted protein; b) obtaining a second vector which expresses all or a functional portion of an ECD of a second TNF-R; and c) ligating the vector of (a) to the vector of (b) via a polypeptide linker resulting in a construct which expresses all or a functional portion of two TNF-Rs linked via a polypeptide sequence. The method can further comprise one or more vectors which express a second polypeptide linker and all or a functional portion of a third ECD of a TNF-R wherein the third ECD of the TNF-R is linked to the second TNF-R via the second polypeptide linker.

The invention further relates to cells which express a receptor molecule which binds to tumor necrosis factor comprising all or a functional portion of the extracellular domain of two or more TNF-Rs linked via one or more polypeptide linker. Suitable cells which can be used to express the receptor molecule include yeast, bacterial and mammalian cells.

The present invention relates to receptor molecules which bind cytokines that bind to receptor molecules comprising more than one subunit. The ECD of such receptors linked by a polypeptide linker have high affinity for the cytokine, and, are effective inhibitors of the biological activity of the cytokine. Thus, the receptor comprises all or a functional portion of the ECD of two or more cytokine receptors linked via one or more polypeptide linkers employing the methods described herein. Thus, the ECD of the receptors of the present invention can be used to inhibit the biological activity of cytokines such as IL-1, IL-2, IL-6, GMCSF, IL-3 and IL-5 (Nicola, N.M. and Metcalf, D., *Cell, 67*:1-4 (1991)).

The invention further includes a method of inhibiting the biological activity of TNF comprising administering to a host an effective amount of a receptor molecule which binds TNF, the receptor comprising all or a functional portion of the ECD of two or more TNF-Rs linked via one or more polypeptide linkers. Such receptor molecules have utilities for use in research, diagnostic and/or therapeutic methods for diagnosing and/or treating animals or humans having pathologies or conditions associated with TNF. Such pathologies can include generalized or local presence of TNF or related compounds, in amounts and/or concentrations exceeding, or less than, those present in normal, healthy subject, or as related to a pathological condition.

For example, the invention includes a method of treating or preventing in a host a TNF related diseases (e.g., autoimmune diseases, inflammatory diseases bacterial, viral or parasitic infections, malignancies and/or neurodegenerative diseases) comprising administering to a host (such as a human) an effective amount of a receptor molecule which binds TNF, the receptor comprising all or a functional portion of the ECD of two or more TNF-Rs linked via one or more polypeptide linkers. For example, the method can be used to treat a host for rheumatoid arthritis, septic shock, cerebral malaria, inflammatory bowel disease, (e.g. Crohn's disease, ulcerative colitis) multiple sclerosis, allograft rejection, graft vs. host disease, neoplastic pathology (e.g., in chachexis accompanying some malignancies) and endotoxemic responses.

The receptor of the present invention can be administered to a host in a variety of ways. The routes of administration include intradermal, transdermal (e.g., slow release polymers), intramuscular, intraperitoneal, intravenous, subcutaneous, oral, epidural and intranasal routes. Any other convenient route of administration can be used, for example, infusion or bolus injection, or absorption through epithelial or mucocutaneous linings. In addition the receptor of the invention can be administered together with other components or biologically active agents, such as pharmaceutically acceptable surfactants (e.g., glycerides), excipients (e.g., lactose), carriers, diluents and vehicles. If desired, certain sweetening, flavoring and/or coloring agents can also be added. The receptor can be administered prophylactically or therapeutically to a host and can result in protection from amelioration of, or elimination of the TNF-related disease state.

Further the receptor molecule can be administered by *in vivo* expression of a polynucleotide encoding the receptor module. The "administration of protein" by definition includes the delivery of a recombinant host cell which expresses the protein in vivo. For example, the receptor molecule can be administered to a host using live vectors, wherein the live vector containing the receptor sequences are administered under conditions in which the receptor molecule is expressed *in vivo*. In addition, a host can be injected with a cDNA or DNA sequence, or a recombinant host cell containing the cDNA or DNA sequence, which encodes and expresses the receptor of the present invention (e.g., ex vivo infection of autologous white blood cells for delivery of protein into localized areas of the body, see e.g., United States Patent Number 5,399,346, which is herein incorporated by reference).

Several expression vectors for use in making the constructs described herein and administering the receptor molecule of the present invention to a host are available commercially or can be reproduced according to recombinant DNA and cell culture techniques. For example, vector systems such as retroviral, yeast or vaccinia virus expression systems, or virus vectors can be used in the methods and compositions of the

present invention (Kaufman, R.J., *J. of Method. in Cell. and Molec. Biol., 2*:221-236 (1990)). Other techniques using naked plasmids or DNA, and cloned genes encapsidated in targets liposomes or in erythrocyte ghosts, can be used to introduce the receptor into the host (Freidman, T., *Science, 244*:1275-1281 (1990); Rabinovich, N.R., *et al., Science, 265*:1401-1404 (1994)). The construction of expression vectors and the transfer of vectors and nucleic acids into various host cells can be accomplished using genetic engineering techniques, as described in manuals like *Molecular Cloning and Current Protocols in Molecular Biology*, which are hereby incorporated by reference, or by using commercially available kits (Sambrook, J., *et al., Molec. Cloning*, Cold Spring Harbor Press (1989); Ausubel, F.M., *et al., Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience 1989)).

An "effective amount" is such that when administered, the receptor molecule of the present invention results in inhibition of the biological activity of TNF, relative to the biological activity of TNF when an effective amount of the receptor is not administered. For example, the inhibition of activity can be at least about 50%, or preferably at least about 75% at the disease site. In addition, the amount of receptor administered to a host will vary depending on a variety of factors, including the size, age, body weight, general health, sex, and diet of the host and the time of administration or particular symptoms of the TNF-related disease being treated. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled in the art. In vitro and in vivo methods of determining the inhibition of TNF in a host are well known to those of skill in the art. Such in vitro assays can include a TNF cytotoxicity assay (e.g. the WEHI assay described in Example 1 or a radioimmunoassay, ELISA). In vivo methods can include rodent lethality assays and/or primate pathology model systems (Mathison *et al., J. Clin. Invest., 81*:1925-1937 (1988); Beutler *et al., Science, 229*:869-871 (1985); Tracey *et al., Nature, 330*:662-664 (1987); Shimamoto *et al., Immunol. Lett., 17*:311-318 (1988); Silva *et al., J. Infect. Dis.,*

162:421-427 (1990); Opal *et al., J. Infect. Dis., 161*:1148-1152 (1990); Hinshaw *et al., Circ. Shock, 30*:279-292 (1990)).

The receptor molecule of the present invention preferably is capable of binding TNF with high affinity. That is, the binding affinity of the receptor molecules described herein for TNF approaches or is greater than the binding affinity of endogenous TNF receptors. Preferably the binding affinity of the receptor is such that the receptor binds the TNF homotrimer in a stoichiometric ratio of about 1:1.

As described in Example 3, the specific activity of the TNF/lymphotoxin inhibitor of the present invention is similar to that of a dimeric p75 TNF-R built on an Ig backbone (Butler, D., *et al., Cytokine, 6*:616 (1994)) and it is therefore capable of inhibiting TNF cytoxicity at a 1:1 molar ratio.

The receptor molecule of the present invention is expected to behave pharmacodynamically as the monomeric TNF-R and be quickly removed from the blood stream via the kidneys (Bemelmans, M.H.A., *et al., Cytokine, 6*:608 (1994); Jacobs, C.A., *et al., Intl. Rev. Exp. Pathol. 34B*:123 (1993)). However, the receptor is expected to have higher penetration to tissues than Ig fusion proteins due to its smaller molecular weight. Preferably, the molecular weight of the receptor molecule of the present invention is about 45 kd to about 130 kd. In addition, the Ig fusion proteins are expected to bind complement to the Fc receptor of a cell surface thereby facilitating development of an immune response. In contrast, the receptors of the present invention, being devoid of an Ig structure, are not expected to be immunogenic.

The invention is further illustrated in the following examples.

EXEMPLIFICATION

Example 1: Cloning of the Hu p75 TNF-R ECD Dimer

In order to express a small molecular weight Hu p75 TNF-R ECD dimer, we constructed the retroviral expression vector, Oscar, that was built in a multiple-step

cloning procedure described below. Plasmids were grown using DH5α competent cells [*supE44 DlacU169 f80 lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*].

PCR of Human p75 TNF-R Extracellular Domain

The Hu p75 TNF-R ECD was amplified by PCR from the pVL1393-Hu p75 TNF-R ECD plasmid using primers (1) and (2) shown below. pVL1393-Hu p75 TNF-R ECD (derived from pVL1393, In-Vitrogen) contained the Hu p75 TNF-R ECD from amino acid 1 to 205 with a 3' stop codon. The 5' primer (1) contained a BamHI restriction site. Bases 7 to 30 of primer (1) annealed to bases 70 to 93 of the mature Hu p75 TNF-R ECD. The 3' primer which anneals to the multiple cloning site of the pVL1393, downstream of the ECD insert, contained an Asp718 restriction site.

(1) 5' TC**GGATCC**CGCCCAGGTGGCATTTACACCC 3'(SEQ ID NO:3) 30mer

(2) 5' CGGAATTCTAGAA**GGTACC**C 3' (SEQ ID NO:4) 20mer

The reaction mix consisted of 0.02mg pDNA, 1mg of each primer, 0.25mM dNTPs, 2.5mM $MgCl_2$, 1xPCR buffer (10x buffer: 500mM KC1, 100mM Tris-HC1 pH 8.3, 0.01% w/v gelatin) and 0.4 units of Taq DNA polymerase to a final volume of 50 µl. The amplification procedure included a denaturation step, 94°C, for 2 minutes, followed by 35 cycles of 1 minute strand separation at 94°C, 1 minute annealing at 56°C, 1 minute extension at 72°C, followed by an elongation step 10 minutes at 72°C.

The extracellular domain (ECD) of the Hu p75 TNF-R ECD with its signal peptide sequence was cloned into the Ncol-Xbal sites of the vector pCITE. pCITE ECD, was derived from pCITE (Novagen) into which the Hu p75 TNF-R ECD, digested from pVL1393-Hu p75 TNF-R ECD with Ncol and Xbal, was cloned. This unit corresponds to the 5' ECD of the final dimer Hu p75 TNF-R ECD. Figure 1 illustrates

the different stages of cloning used to obtain the Hu p75 TNF-R ECD dimer. Also shown in Figure 1 are the principal restriction enzymes sites of Hu p75 TNF-R ECD.

Cloning of the 3' Hu p75 TNF-R ECD Into pIg16

The 3' ECD was first amplified by PCR to introduce a 3' stop codon and two unique restriction sites at either end for cloning into the plasmid pIg16 which contains a single chain Fv anti-DNA antibody cloned in it. The plasmid pIg16 (Brigido, M.M., *et al., J. Immunol., 150*:469 (1993)), derived from the pGEM-3Zf(-) vector (Promega) and containing a scFv construct was obtained from Professor David Stollar, Tufts University.

The PCR reaction product was phenol extracted, ethanol precipitated, resuspended and its ends blunted with Klenow fragment of DNA polymerase. The DNA was phenol extracted, ethanol precipitated, resuspended and digested with BamH1 and Asp718. The 770bp product was purified by agarose gel electrophoresis, reprecipitated and ligated into the BgIII/Asp718 sites of pIg16.

The 3' ECD cloned into pIg16, replacing the VL domain from this construct, was named p75s. The product, p75s, was confirmed by restriction analysis and contained the Hu p75 TNF-R ECD, with a 3' stop codon, immediately downstream of the pIg16 polyglycine linker sequence (Brigido, M.M., *et al., J. Immunol., 150*:469 (1993)).

Construction of Dimeric Hu p75 TNF-R ECD Retroviral Vector

The polyglycine-serine linker and 3' ECD were removed together from p75s and cloned into pUC18 in tandem with the 5' ECD from the pCITE-ECD construct. pUC18 was obtained from Pharmacia.

p75s was digested with XbaI, the 5' overhangs filled in with Klenow and digested with Asp718. The 800bp fragment was purified by agarose gel electrophoresis, precipitated and resuspended in water.

pCITE ECD was digested with EcoRI and PvuII removing the Hu p75 TNF-R ECD with its signal peptide and CITE sequence. The 1500 bp fragment was purified by agarose gel electrophoresis, precipitated and resuspended in water. These two fragments were ligated into the EcoRI/Asp718 sites of pUC18 to produce the Hu p75 TNF-R ECD-dimer construct, TRIP-4, confirmed by restriction analysis.

The Hu p75 TNF-R ECD dimer construct was removed from the pUC18 vector and placed into the retroviral vector pBabeNeo, the clone obtained was named Oscar. TRIP-4 was digested with NcoI, the 5' overhang filled with Klenow and digested with SalI. The 1600bp fragment was purified by agarose gel electrophoresis. The fragment was ligated into the retroviral vector pBabeNeo (Morgenstern, J.P. and Land, H., *Nucleic Acids Res., 18*:3587 (1990)) which had been digested with BamHI, blunted with Klenow, and digested with SalI. pBabeNeo contains a MuLV LTR promoter, a neomycin resistance gene under the control of an SV40 promoter and an ampicillin gene. The Hu p75 TNF-R ECD dimer was inserted into the multiple cloning site 3' to the gag gene and 5' to the SV40 promoter (Figure 2). The resulting clone, named Oscar, was confirmed by restriction analysis.

The open reading frame of the soluble Hu p75 TNF-R ECD dimer with its polyglycine-serine linker is shown in Figure 3.

Example 2: Transfection of GPenvAM12 Cells with the Dimeric Hu p75 TNR-R ECD Retroviral Vector

Permanent transfections were done in GPenvAM12 cells (Markowitz, D., *et al.*, *Virology, 167*:400 (1988)). Stable transfectants expressing the Hu p75 TNF-R ECD dimer were made in the cell line GPenvAm12 and G418 was used to select for permanent transfectants. These cells constitutively express the protein which is secreted into the media.

The GPenv AM12 cells were grown and maintained in DMEM medium supplemented with 10% new-born calf serum, 2.5 units/ml penicillin, 2.5 µg/ml streptomycin and 2 mM glutamine.

For stable expression of Oscar from GPenvAM12 cells (Markowitz, D., *et al.*, *Virology, 167*:400 (1988)), 20 µg of vector DNA were transfected into the cell line using the calcium-phosphate precipitation method. Transfected cells were selected and maintained in medium with 1mg/ml G418. G418 resistant cell clones were pooled and tested for expression of Hu p75 TNF-R ECD dimer by ELISA, Western and inhibition of the TNF cytoxicity assay on WEHI cells.

To collect the secreted dimer from the supernatant of the stable transfected cell line, cells were grown to 80-100% confluence in the presence of 0.5 mg/ml G418. The media was removed and the cells washed twice in serum-free media. Fresh serum-free media was added to the cells, without G418, and the supernatants and cells harvested after 48 hours. Supernatants were stored at -70°C until used.

ELISA Assay

Concentrations of Hu p75 TNF-R ECD, produced by transfected GPenvAm12 cells, were determined by ELISA. The monoclonal antibody 4C8 (Dr. Buurman, Maastricht, The Netherlands) was used as trapping antibody and the ELISA assay performed as described (Bemelmans, M.H.A., *et al.*, *Cytokine, 6*:608 (1994)). A titration curve was prepared with a standard Hu p75 TNF-R ECD diluted 1:1 in PBS, 0.1% BSA at concentrations ranging from 62 pg/ml to 5 ng/ml. The amounts secreted averaged 560 pg/ml (3400 pg/plate) and were too low for immediate detection by Western blot analysis.

Western Blot

The serum-free medium from the GPenvAM12 cells was concentrated by centrifugation using Amicon Centricon 30 concentrators. The concentration of the soluble TNF inhibitors were determined by ELISA.

9% SDS-PAGE were run to standard western protocol and probed using the monoclonal antibody 4C8 to the Hu TNF-R75 ECD and a polyclonal anti mouse secondary antibody crosslinked with horseradish peroxidase. Westerns were developed using the ECL detection system (Amersham).

Each slot contained from left to right: 0.5 ng of dimeric Hu p75 TNF-R ECD, GPenvAM12 control supernatants 1 and 2, 1 μg soluble p75 sf2 Ig dimer (Butler, D., *et al.*, *Cytokine, 6*:616 (1994)) and 8.7 μg soluble hs p75 TNF-R CRIP monomer. These were separated on a 9% acrylamide gel, electroblotted onto nitrocellulose, probed with 4C8 monoclonal antibodies and HRP-linked secondary antibodies and developed using the ECL system.

After concentration of the supernatants to 20 ng/ml, the Hu p75 TNF-R ECD dimer was clearly detected in the supernatant of Oscar stable tranfectants as a band of apparent molecular weight of 59 kDa (Figure 4, left lane). The arrow indicates the sTNF-R dimer with apparent molecular weight of 59 kDa. The positions of molecular weight markers are indicated on the right. The band, detected by the monoclonal antibody 4C8 was not present in the GPenvAm12 untransfected cell supernatants. The expected molecular weight of the dimer was 53 kDa although there are four potential N-linked glycosylation sites within the Hu p75 TNF-R ECD protein (Figure 3). This glycosylation sites may explain the increase in apparent molecular weight.

The Hu p75 TNF-R ECD dimer protein seems to be stable to proteolytic degradation since no smaller products were detected especially when compared to the Ig- fusion protein p75 sf2 (Figure 4). The smaller difference seen between the monomeric 40 kDa (Figure 4, right lane) and the dimeric 59 kDa dimeric Hu p75 TNF-

R ECD (Figure 4, left lane) is probably due to secondary structure obtained by the presence of the polyglycine-serine linker.

Example 3: Protection from TNF Cytotoxicity on WEHI cells by Hu p75 TNF-R ECD construct

WEHI Assay. The concentrated supernatants were tested for protection against TNF cytotoxicity in the WEHI cell assay. To measure the inhibitory effect of the expressed Hu p75 TNF-R ECD dimer on TNF cytotoxic activity, WHEI 164 clone 13 mouse fibrosarcoma cells were used (Espevic, T., and Nissen-Meyer, J., *J. Immunol. Methods, 95*:99 (1986)).

Figure 5 shows the protective effect obtained in this assay when TNF was preincubated with dilutions of various Hu p75 TNF-R ECD proteins. However, the two dimeric Hu p75 TNF-R ECD constructs namely p75 sf2 and Oscar efficiently protected WEHI cells from TNF cytotoxicity. Table 1 shows that 20 pg dimeric Hu TNF-R75 ECD were sufficient to inhibit by 50% the killing activity of 63.5 pg of human TNF. In comparison, 57 pg of the dimeric Hu p75 TNF-R ECD in an Ig backbone (p75 sf2) (Butler, D., *et al.*, *Cytokine, 6*:616 (1994)) were needed to obtain the same level of protection. This lower than expected activity of the p75 sf2 construct may be due to the partial degradation in this protein (Figure 4) that affected its efficiency. The monomeric Hu TNF-R75 ECD at 300 fold higher concentration was not effective at blocking TNF cytoxicity in the WEHI assay (Figure 5). The cell line CRIP producing monomeric Hu p75 TNF-R ECD, was provided by Dr. Paul Robbins, University of Pittsburgh.

The concentration of 20 pg/ml Hu p75 TNF-R ECD dimer needed to inhibit by 50% the cytotoxic effect of 62.5 pg/ml TNF indicates that this antagonist is capable of binding to the TNF homotrimer in a stoichiometric ratio of almost 1:1.

Table 1

Specific activity of Hu p75 TNF-R ECD dimer

	Mr (kD)	50% protection
OSCAR (Hu p75 TNF-R ECD dimer)	59,000	20 pg/ml
hs p75 TNF-R CRIP (Hu p75 TNF-R ECD monomer)	40,000	N/A
IgG-ECD (Hu p75 TNF-R ECD dimer on Ig)	150,000	57 pg/ml

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.